RESEARCHREPORT

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SWINE HEALTH

Title: Rational Design of a New Generation of PRRSV Differential (Marker) Vaccines (Renewal Summer 2006) - **NPB #: 06-177**

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Abstract

This project deals with the development of a new generation of PRRSV differential marker vaccines. We are successfully applying the technology of reverse genetics [infectious clone] to dissect the function of many different parts of the genome and genes in the life cycle of PRRSV in a host. The project is based on the notion that the best type of immunogen against PRRSV has proved to be, so far, a (live, replicating) vaccine that presents the antigens to the pig's immune system in a similar way as wild-type PRRSV does. Therefore our ultimate goal is to develop a live, replicating vaccine of safety and efficacy that would be compatible with eradication. The proposal's objectives are addressed to find answers to the following specific questions**: 1. What is the molecular basis of attenuation of virulence in PRRSV? 2. Can we molecularly attenuate PRRSV and obtain a replicating vaccine of unprecedented efficacy and safety? 3. Can we engineer this product to be a "marker" vaccine so that we can integrate robust vaccination together with efficient eradication of PRRSV**? A last, fundamental long-term point requiring an answer is the design of this novel vaccine up to standards of satisfactory protective coverage against infection by both homologous or heterologous PRRSV strains. With these goals in mind we are carrying out, as reported here, a systematic molecular characterization of the virulent phenotype of PRRSV so as to identify the genetic markers of virulence in PRRSV and precisely engineer an attenuated vaccine candidate. We are also defining a series of small segments of PRRSV proteins (epitopes) that can be deleted from the vaccine that could then be used as serologic diagnostic markers of infection. Under this research we have recently reported the construction of the first, proof-of-concept, DIVA (differentiating infected from vaccinated animals) live PRRSV strain that could be use as base platform of a novel marker MLV vaccine X PRRSV.

These research results were submitted in fulfillment of checkoff funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer reviewed

III. Introduction

The significance of a new generation of PRRSV differential vaccines is well exemplified by the prominent rank always given by industry to this topic. Since the launching of the PRRSV Initiative in 2003, the topic of new PRRS vaccines has been high in the list of research priorities for this NPB PRRSV research grant program. Furthermore, a joint review of the PRRSV Initiative components (NPB and CAP-USDA programs) conducted in 2006 has confirmed such priority and defined that, within the general area of "PRRSV vaccines", the four major priorities identified by industry are: **1. To develop (a) vaccine(s) that produce(s) complete heterologous protection. 2 Evaluate candidate vaccines using the sow model. 3 (..Determine…) what are the alternative strategies for vaccine development, and 4. (..Determine..) What are the genetic determinants for virulence attenuation.** More recently, on June 1st, 2007, a meeting (Title: **Colloquium on Prospects for Development of an Effective PRRS Virus Vaccine**) was held at the University of Illinois College of Veterinary Medicine to discuss the state of current knowledge about PRRS vaccination. The meeting was attended by 26 invited experts in PRRS, virology, immunology and vaccinology and included clinical veterinarians, academics and vaccine industry scientists. The main conclusions of this comprehensive meeting on PRRSV vaccines were:

- PRRS vaccines are effective against homologous challenge.
- Current vaccines are not adequate for producer needs.
- Important research questions that need to be addressed to improve PRRSV vaccines include: 1) to define all of the PRRSV components which have a role on induction of protective immunity 2) to pursue the mapping of T and B cell epitopes significant for the induction of protective immunity 3) fully understand the mechanisms of both homologous and broad (syn: heterologous) protection 4) identify the genetic determinants /phenotypes of virulence/attenuation, host range and immune evasion and how they relate to vaccine.
- Improved PRRSV vaccines should be available in 5-10 years.

In summary, vaccine improvement is a reachable goal within the technical capabilities currently available. In full agreement with the goals defined by the experts' symposium, this project represents efforts directed to the betterment of current modified live vaccines by generation of live PRRSV vaccines that have been attenuated in a molecular way, with increased immunogenicity obtained by molecular modification, and counting with the addition of molecular markers for DIVA differentiation through a simple serologic test.

IV Objectives

This entire project was anticipated to require a total of 30 months for full completion At the time of submission of the NPB 06-177 we requested support for one year of work covering the following 4 objectives:

No	Objective
1	To construct molecularly defined PRRSV attenuated mutants and characterize them biologically
2	To test the molecular enhancement of immunogenicity in existing MLVs
3	To define non-essential regions of the PRRSV genome that could be used as serologic indicators of infection
4	To test the fitness of the marker antigen as a serologic test that would be used in herds

V. Materials and Methods:

Construction of full-length chimeric cDNA clones

We used chimeric constructs made between two (one virulent and another attenuated) strains of PRRSV to map the virulence markers of PRRSV. Full-length FL12 infectious clone (wt) and PP18 infectious clone (Primepac, PP, attenuated strain infectious clone) were utilized as backbones to construct chimeric clones. First, to make chimeric clones covering multiple genes derived from the PP vaccine virus strain, a series of PCR products, which contain naturally present or deliberately introduced restriction endonuclease (RE) sites, RsrII, SpeI, MluI, Pmel, SgfI, EcoRV, BssHII, BstBI and PacI, were used to replace the corresponding regions of FL12 clone as described in more detail in our paper (Kwon et al. 2006) . In addition, the construction of single gene chimeric clones comprising individual structural protein encoding genes from either the PP vaccine virus or vFL12, additional RE sites, BsrGI, BstEII and NruI were incorporated to facilitate the fragment exchange in a similar manner. The target genes were either directly cloned into full-length FL12 or into an intermediate plasmid encompassing the majority of ORF2, complete ORFs 3 to 7, and the entire 3' UTR derived from the FL12 as described In our paper (Ansari et al. 2006). Likewise the reciprocal chimeric clones containing either ORF2 or ORF5, and ORFs 2 and 5 of FL12 were prepared in the genetic background of PP18 clone (Fig. 2) by using the same (BssHII and BstBI) and alternative (SacII) RE sites. All generated chimeric cDNA clones were confirmed by sequence analyses of the corresponding fragments.

Rescue and analysis of chimeric viruses

In vitro transcription, RNA electroporation, assessment of virus rescue by immunofluorescence assay, and characterization of viral growth properties and kinetics in MARC 145 cells is fully described in our published papers (De Lima et al., 2006, Ansari et al. 2006, Kwon et al., 2006)

Animal experiments

Pregnant sows were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. Their negative PRRSV infection status was confirmed upon arrival by a commercial ELISA serology test (IDEXX Labs, Portland, ME). Pregnant sows were inoculated at 90 days of gestation and the viability scores of off-springs at birth and weaning (at 15 days of age) were evaluated. All animals were tested for anti-PRRSV antibodies by ELISA (IDEXX Labs, Portland, ME). Two sows per chimera in each separate experiment were infected with parental and chimeric viruses. In all the cases, a total of 2 ml ($10^{5.2}$ $TCID_{50}$) of virus was administered intra-nasally with 1ml into each nostril. The rectal temperature and clinical signs of the inoculated animals were monitored daily from 3 days pre-inoculation through farrowing and to weaning. Viremia was measured by regular titration on MARC-145 cells and by nested RT-PCR , using the sera collected between 0-14 PI days PI. In some cases, copy numbers of viral RNA from the sera of sows were measured using a PRRSV real-time quantitative PCR kit (Tetracore, USA).

Two groups of fifteen mixed-breed (Landrace x Large White) piglets averaging 3 weeks of age obtained from a PRRSV-free farm were allocated in three BL-2 isolation rooms. At the beginning of the experiment, all animals were tested negative for PRRSV-specific antibodies as measured by a commercially available ELISA kit (IDEXX Labs, Inc). The animals from the experimental groups were inoculated with a total dose of 105.0 TCID50/3ml of PRRSV FL12 or the epitope deletion mutant (FLdNSP244) by intranasal (1ml in each nostril) and intramuscular (1ml) routes. The inoculated animals were clinically monitored on a daily basis and their rectal temperatures were recorded from day 3 pre-inoculation to day 15 post-infection (pi). Sequential blood samples were collected from all animals at days 0 (zero), 7, 15, 30, 45 and 60 pi. In order to assess the virulence of the epitope deletion mutant (FLdNSP2/44), we inoculated two pregnant sows at day 90 of gestation by intranasal route with 105.0 TCID50/ml of the mutant virus to evaluate the viability scores of the offspring at

birth and weaning. The sows were acquired from a specific-pathogen free herd with a certified record of absence of PRRSV infection. Inoculated sows were clinically monitored from 3 days prior to inoculation until 15 days post-farrowing. Blood samples were collected at days 0 (zero), 7, 15, 30, 45 and 60 pi for serological and virological analysis.

Introduction of deletions into the full-length PRRSV cDNA infectious clone

In order to explore deletions of possible markers, two regions were previously selected for deletion in the NSP2. Each region spans 15 amino acids residues which were found to be highly immunogenic, relatively conserved among US-type PRRSV strains and most importantly, consistently recognized by antibodies of PRRSV-infected pigs [12]. The regions selected as serological marker candidates were P431PPPPRVQPRKTKSV445 and K441TKSVKSLPGNKPVP455 within NSP2 amino acid sequence. All deletions were introduced into the pFL12 plasmid which contains the full-length cDNA of NVSL 97-7895 PRRSV strain [14], by overlap extension method as previously described [16]. Sequences from either side of the point of deletion were amplified by using specific primers designed such that their 3'ends hybridize to template sequence on one side of deletion and the 5' ends are complementary to template sequence on the other side of the deletion (Table 1). Using this approach the products generated from the PCR reaction using the reverse and forward primers with overhang regions are therefore overlapping at the deletion point. After amplification of the flanking regions, both amplicons were purified from a low melting point gel and precipitated by phenol-chloroform using standard protocol as described elsewhere [17]. Gel-purified DNA were mixed equally and submitted to 4-5 cycles of PCR followed by addition of external primers. After gel purification and precipitation the DNA was digested with SpeI and SphI or XhoI and the fragment was cloned directionally into the pFL12. Confirmation of the deletion and absence of any other mutations within the region was confirmed by nucleotide sequencing.

Rescue and analysis of the viable deletion mutants obtained upon transfection of MARC 145 cell were conducted following the same protocols as the chimeric mutants.

Kinetics of virus growth

MARC-145 cells were infected either with FL12 or FLdNSP2/44 at an MOI of 0.1 TCID50 per cell and incubated at 37°C in 5% CO_2 atmosphere. Aliquots of culture supernatants from infected cells were collected at different time points (0, 6, 12, 24, 48 and 72 hours post-infection) and the virus titer was determined by 50% end-point analysis and the titers were expressed as tissue culture infectious dose 50 per ml (TCID50/ml). The viral growth kinetics assays were performed in triplicate.

Peptide ELISAs

Serum samples collected until day 60 pi from all piglets experimentally infected with FL-12 (wild-type strain) and FLdNSP2/44 (epitope deletion mutant) and from the sows infected with the PRRSV deletion mutant were tested using a peptide-based ELISA for screening of the peptide-specific antibody response, as previously described [12]. Briefly, Immulon 2HB flat bottom microtiter 96 well plates (Thermo Electron, Milford, MA) were coated with 100µl of a peptide [P431PPPPRVQPRKTKSV445] solution (10µg/ml) in 0.1M carbonate buffer (pH 9.6), and incubated overnight at 4ºC. After blocking with 250µl of a 10wt. % nonfat dry milk solution for 4h at room temperature on a plate shaker, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST-20). Unbound reagents were further removed by striking the plates repeatedly, bottom up, on a stack of absorbent paper towel. Then, 100µl of pig sera (1:20) diluted in 5wt. % nonfat dry milk in PBST-20 was added per well and plates were incubated in the shaker for 1h at room temperature. After washing five times with PBST-20, each well received 100µl of the affinity purified antibody peroxidase labeled goat anti-swine IgG (KPL, Gaithersburg, MD) diluted 1:2000 in PBST-20 with 5wt. % nonfat dry milk for and the plate was incubated for 30min at room temperature. Following a final wash, 100µl of ABTS (KPL) peroxidase

substrate was added for 15 min at 37ºC and the reaction was stopped by adding 100µl of 1% SDS. Sera were considered positive when the OD value was above the cutoff point (the mean OD absorbance at 405nm of the negative sera plus 3 standard deviations).

Sequence analysis

Multiple alignment of nucleotide and amino acid sequences obtained either from Gene Bank data base or from the sequencing facilities that analyzed our mutants were made using ClustalW [18]. Alignments were retrieved and analyzed by Bio-Edit sequence alignment editor v.7.0.5.

VI. Results:

Objective 1:

In order to determine virulence-associated genes in porcine reproductive and respiratory syndrome virus (PRRSV), a series of chimeric viruses were generated where specific genomic regions of a highly virulent PRRSV infectious clone (FL12) were replaced with their counterparts of an attenuated vaccine strain (Prime Pac). Initial genome-wide scanning using a sow reproductive failure model indicated that non-structural (NSP3-8) and structural (ORF2-7) genomic regions appear to be sites where major virulence determinants of PRRSV may reside. In addition, two other non-structural regions (NSP1-3 and NSP10-12) possessed an intermediate attenuation phenotype. These results thus confirm the multigenic character of PRRSV virulence. Additional chimeras containing each individual structural ORFs (2 through 7) of Prime Pac and ORF5 of Neb-1 (parental strain of Prime Pac) within the FL12 backbone were generated and tested individually for further mapping of virulence determinants. Our results led to the conclusion that ORF5, encoding glycoprotein 5 (GP5), is the major structural determinant of PRRSV virulence in pregnant sows. The ORF2 may also contribute to virulence, although to a lesser extent.

Objective 2:

By inoculation of the doubly deglycosylated GP5 mutant PRRSV strains in sows we have observed that the stability of the deglycosylated strains in vivo may not be permanent and that the mutant may tend to revert to recover a compensatory glycosylation in the genome , thus originating mixed ambiguous results regarding the enhancement of SN . We confirmed this observation by full genomic sequencing and glycolsylation phenotype of the mutant recovered from individual animals at 15 days pi, (serum sample positive for viremia). We have started joint experiments with the biologics company Avimex-Mexico, to study the stability of the deglycosylated forms of GP5 (double and triple deglycosylated forms OF GP5) in the context of a swine adenovirus vector and co-expressed with M protein as a hetero-dimer. It is possible that the deglycolsylated form of GP5 may be more stable when used as a foreign antigen insert in a replicating vector rather than as a live replicating molecule of PRRSV RNA.

Objectives 3 and 4:

A mutant PRRSV lacking an immunodominant B-cell linear epitope of Nsp2 in a North American strain of PRRSV was successfully generated by reverse genetics using our infectious clone pFL12. Removal of the 15-mer Nsp2 epitope had no effect on growth properties (figure 1) , imunogenicity (figure 2, data obtained by Idexx ELISA) or virulence in piglets (table 1) or sows (table 2) . Pigs inoculated with FLdNsp2/44 did not develop antibodies directed against the selected epitope as measured by a peptide-based ELISA (figure 3). The combination of a mutant virus carrying a deletion of an immunodominant epitope of Nsp2 and the corresponding peptide ELISA proves that epitope deletion can endow PRRSV live attenuated vaccines with DIVA differential capability. While the NSP2 epitope used for deletion in these experiments to generate

FLdNSP2/44 was definitely recognized as immunodominant in our previous research (de Lima et al .Virology 353: 410-421), evaluation of field sera with the peptide-specific ELISA indicates that the level of conservation of this particular NSP2 epitope is low, with just about 25 % of field sera from wt PRRSV-infected animals exhibiting positive reactivity against this epitope (unpublished research). Therefore, even if this epitope is immunodominant, thus inducing antibody response in the majority of animals infected with the same strain, its level of conservation among PRRSV strains seems relatively low or suboptimal. Efforts should now continue towards identifying the optimal marker, capable of reliably inducing a detectable serologic response in the diverse universe of wt PRRSV strains. To that end we should pursue the development of PRRSV mutants carrying amino acid substitutions within a highly immunogenic and highly conserved epitope of M protein (ep. # 201, de Lima et al .Virology 353: 410-421). Such approach might eliminate its immunogenicity without impairing other fundamental biological functions of the protein which may help rescue viable virus. Initial deletion of previously identified epitopes of Gp3 and M protein were lethal for virus recovery, although amino acid substitutions within epitope #201 (ORF6) rescued viability but did not eliminate its immunogenicity. Regarding this marker, further fine mapping of epitope #201 should follow employing substitutions of every 2-3 aminoacids

VII. Discussion

A. Findings on PRRSV virulence and attenuation

By systemically exchanging genomic regions and generating a series of chimeric viruses containing the whole genome of PRRSV, specific non-structural and structural regions associated with virulence were confirmed. Recently a similar approach was reasonably successful in mapping of virulence genes in several viruses (swine vesicular disease virus, foot-and mouth disease virus,, classical swine fever virus, porcine circovirus type 2 and type 1, simian immunodeficiency virus and vaccinia virus).

Based primarily on the sow reproductive failure model, we conclude that the ORF5 structural gene plays a major role in determining virulence of PRRSV. Likewise, the ORF2 gene would also have a measurable but lesser role in virulence. The ORF5 of the viral genome encodes the major viral envelope glycoprotein 5 (GP5), which is a glycosylated trans-membrane protein of approximately 25 kDa . Like EAV and LDV, the PRRSV GP5 and matrix (M) protein interact and form heterodimers, which may play a critical role in virus assembly but whose mode of interaction has not been demonstrated yet. The GP5 is believed to be involved in the entry of virus into the host cells, presumably by interacting with the host cell receptor, especially for macrophages. The GP5 is considered to be important in the infection process because the presence of a major neutralization epitope in the N-terminal ectodomain may be involved in receptor recognition. Moreover, our results provide evidence that glycosylation of GP5 of PRRSV plays an important role for immune evasion and persistence to escape or minimize virus-neutralizing antibody response by the glycan-shielding mechanism. This glycan shielding mechanism is also important for LDV, HBV, SIV, Influenza, and HIV . The ORF2 of the PRRSV genome encode 2 different envelope proteins, one minor glycosylated glycoprotein 2a (GP2a) of 29~30kDa and the other non-glycosylated small membrane protein, 2b of 10kDa. Compared to GP2a, 2b is predominantly expressed in infected cells. In EAV, GP2b (GP2a of PRRSV) interacts with other glycoproteins GP3 and GP4 by forming intra- and inter-molecular disulfide bonds and the correct association is important for their efficient incorporation into viral particles and for virus infectivity and the protein E (2b of PRRSV) is required for the production of infectious virions. However the protein 2b of PRRSV has been shown not to interact with other proteins and the cysteine residues are not essential for virus replication .Future work will continue to uncover the precise role of single residue(s) for virulence within ORFs 2 and 5 genes by site-directed mutagenesis followed by in vitro and in vivo characterization. In addition, more virulence determinants will be identified within NSP3 to 8 regions, which appears to represent a very strong cluster of virulence genes.

The methodology used by us until now, based on preparing chimeras between related but non-homologous PRRSV strains may pose limitations due to the non-natural differences represented by external addition of sequences added to our infectious clone to facilitate cloning of the chimeric segments, increasing significantly the number of aaminoacid residues to compare and study by mutagenesis in order to identify the true markers

for virulence. Alternatively, these potential complications may be obviated by switching our work to a recently identified, alternative pair of parental wild-type /attenuated vaccine PRRSV strains. Such wt parent-vaccine derivative pair has been identified by routine analysis of PRRSV sequence data bases in our laboratory (De Lima et al, 2007, unpublished results). The pair exhibits very high homology with our IC FL12 strain. Such pair is composed by two well known strains: Ingelvac ATP (a current commercial vaccine strain that has a well characterized attenuated phenotype) and JA142 (its parental wild-type from which it was derived Ingelvac ATP).We have been able to confirm that the JA142 and 97-7895 (the parental strain of our FL12 IC) strains maintain very close genetic homology (to the level of being nearly identical at about 99.9 %,). The close identity between these two strains can be explained because both strains (JA142 and 97-7895) were isolated very closely both in time and location of outbreak (Dec 1996 and January 1997) from nearby farms in SE Iowa. For all practical purposes concerning the future of this project, our FL12 IC can be considered a *de facto* IC for the JA142 wt strain. The close genetic homology between JA142 and our FL12 IC [i.e: only 5 nucleotides difference between FL12 and JA142 throughout the whole genome] could facilitate the identification of aa residues involved in attenuation/virulence when compared to Ingelvac ATP strain. A whole genome sequence of Ingelvac ATP, to our knowledge not yet available, can be easily obtained by means of our own set of primers in use in our laboratory for FL12/97-7895 full genome sequencing, which in all likelihood will be identical or adaptable to Ingelvac ATP sequencing with minor modifications. Such analysis would permit the comparison of sequences between attenuated and virulent strains under conditions of significantly higher homology background than we have been operating until now.

B. Development of a DIVA marker strain of PRRSV

These experiments resulted in the generation of a PRRSV carrying a deletion of an immunodominant B-cell linear epitope contained in the NSP2 gene. This epitope had been previously identified as being consistently recognized by the antibody response in PRRSV-infected animals . The successful recovery of the epitope deletion mutant (FLdNSP2/44) and its active replication in pigs, demonstrates that this specific region is dispensable for virus replication in vitro and in vivo. Previous reports have shown that the NSP2 replicase protein of PRRSV tolerates insertions, and deletions, thus representing an ideal target for the development of marker vaccines . Most importantly, our mutant exhibited an efficient growth in vitro and in vivo and the induction of specific seroconversion as measured by a commercial ELISA kit, with absence of a marker-specific peptide-ELISA response in 100% (n=15) of the inoculated animals, thus fulfilling the requirement for DIVA marker differentiation.

To test whether the epitope deletion made on the mutant had altered the general biology of the strain, we examined the in vivo phenotype of the epitope deletion mutant by inoculating two pregnant sows at day 90 of gestation. Infection of pregnant sows with virulent PRRSV strains, specifically with our wt infectious clone FL12, invariably results in abortion, mummified or stillbirth piglets being a reproductive failure model to study virulence. Infection of the pregnant sows with FLdNSP2/44 resulted in abortion in similar levels when compared to the wild type virus indicating that the removal of the 45 nucleotides from NSP2 did not result in attenuation. Although the absence of virulence will be an essential requirement for the final live virus vaccine candidate, we did not expect that any significant level of attenuation could be attributed to the 15 amino acid deletion.

While the development of this mutant FLdNSP2/44 supports the concept of development of live PRRSV strains that can be differentiated serologically by a specific peptide ELISA, the particular marker that we describe in NSP2 still falls short of being the ideal DIVA marker candidate for PRRSV vaccines. Efforts are therefore now concentrated on obtaining deletions or substitutions of the most conserved markers previously reported, which are based mainly in regions located at the endodomain of the M protein. In this case a particular challenge is posed by the fact that this well-conserved epitopes are not amenable to deletion, thus obliging us to attempt the

elimination of the antigenicity of the marker sequence without losing viability of the strain using single point mutations.

In summary, in vitro and in vivo characterization of the FLdNSP2/44 showed that removal of a 15-mer NSP2 epitope had no effect on immunogenicity, growth properties or virulence of the mutant virus. In addition, pigs inoculated with FLdNSP2/44 did not develop antibodies to the selected epitope as measured by a peptide-based ELISA, whereas a strong reactivity was observed in the sera derived from animals infected with the wild-type virus (FL12). Taken together, our results provide proof of concept demonstrating the feasibility of constructing a PRRSV live-attenuated marker vaccine by deleting an immunodominant B-cell linear epitope from the vaccine strain. In addition, the combination of a mutant virus carrying an epitope deletion and its corresponding peptide-based ELISA represents an attractive approach for the development of PRRS differential vaccines. As this NSP2 epitope maker would be suboptimal for widespread detection of PRRSV wt strains due to the low conservation and high variability of NSP2, efforts should now focus on identifying an optimal marker such as those in PRRSV M endodomain, currently under investigation in our laboratory.

VIII. Lay Interpretation:

This project is based on two main premises: 1) the conviction that the use of vaccines will always be a cost-efficient method and the preferred approach to control PRRSV infections, and 2) the notion that the best type of vaccine against PRRSV has proved to be the modified live, attenuated vaccines.

In all likelihood, the live vaccines are most effective because their components that are determinants of protection (a.k.a. as antigens or immunogenic epitopes) are "seen" by the pig's immune system in a similar way as are seen those of live wild-type (fully infectious) PRRSV. A nationwide colloquium held by PRRSV experts in June 2007 at the University of Illinois (Urbana-Champaign) reached the conclusion that a new vaccine for PRRSV may require up to 10 years to reach the market, and that several technical approaches may be followed to develop such novel vaccine. However, the expert group concluded that the live, replicating type of vaccine seems generally to be the most favored, based on the robust immunity that can be developed in the animal after the application of this type of vaccine.

Our ultimate goal is to develop a live vaccine of safety and efficacy that would be compatible with the ability of cleansing the PRRSV infection, that is, compatible with the ability of differentiating, through a simple test, the vaccinated/protected animals from those that have suffered infection by wild-type PRRSV. Our technical approach to the improvement of PRRS live vaccines is based on the notion that mapping the genes causing virulence in PRRSV should provide information for the development of a differential PRRSV vaccine of unprecedented safety and efficacy. The capacity of PRRSV to cause serious pathologic changes is called virulence. In our system we measure viral virulence in relation to the virus' ability of producing abortion in pregnant sows. This virulence is caused by the different genes and its proteins composing the PRRSV. The main expected outcome of this research is the alteration of the genes of the PRRS viruses to develop live attenuated/marker vaccine strains. The research we report herein uses chimeras, which are strains obtained by the mixing of genes of two heterologous PRRSV strains of different level of virulence. Engineering of new live-attenuated PRRSV marker vaccines requires knowledge of the genetic make-up of PRRS virulence and identifying small areas of the proteins which can be eliminated from the vaccine without affecting the virus' ability to multiply in the pig. This concept is similar to that applied for Pseudorabies marker vaccines. The differential vaccines, which, like in the example of Pseudorabies, were originally called "marker vaccines" are now also identified as DIVA vaccines (which stands for "Differentiating Infected from Vaccinated Animals"). This year, through the support from NPB #: 06-177, we are able to report the development of the first DIVA live vaccine for PRRSV through the elimination of small protein fragment (epitope) that, although falling short of being a perfect marker, serves as proof of the concept and encourages further research on more efficient small protein fragments that can be used as markers. Likewise we report that PRRSV virulence, while evidently

is under control of more than one, perhaps several, genes, can be irreversibly modulated by changes in just a few important PRRSV genes, one of which is GP5. This knowledge and the realization that the virulence studies can be continued through the use of a recently identified homologous pair (virulent wt and vaccine strain pair), without using chimeras, will significantly speed the characterization of the minimal number of segments of PRRSV genes that need to be altered to obtain a safe (non-virulent) efficacious vaccine candidate.

Publications in refereed journals

This project (NPB 06-177) has provided support for the following two publications submitted [a] or published [b] in refereed journals:

(a) Identification of virulence determinants of Porcine Reproductive and Respiratory Syndrome virus through construction of chimeric clones Kwon B, Ansari IH, Pattnaik AK, and Osorio FA, submitted to *Virology* June 15, 2008

(b) de Lima M, Kwon B, Ansari IH, Pattnaik AK, Flores E F , and Osorio FA.2008. Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes *Vaccine* 26: 3594-3600

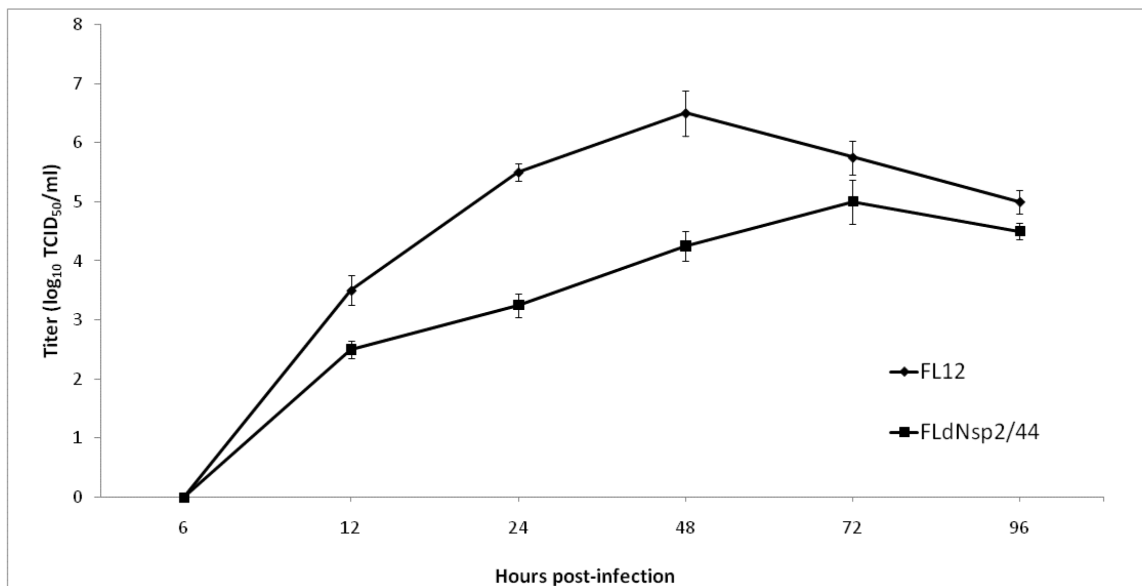

Fig. 1. Growth kinetics of the epitope deletion mutant (FLdNsp2/44) and its parental virus strain (FL12). MARC-145 cells were infected at an MOI of 0.1 and culture supernatant was collected at different time points after infection. Virus infectivity was calculated by 50% end-point titration according to the Reed and Müench method. Results represent the mean values obtained in three independent experiments.

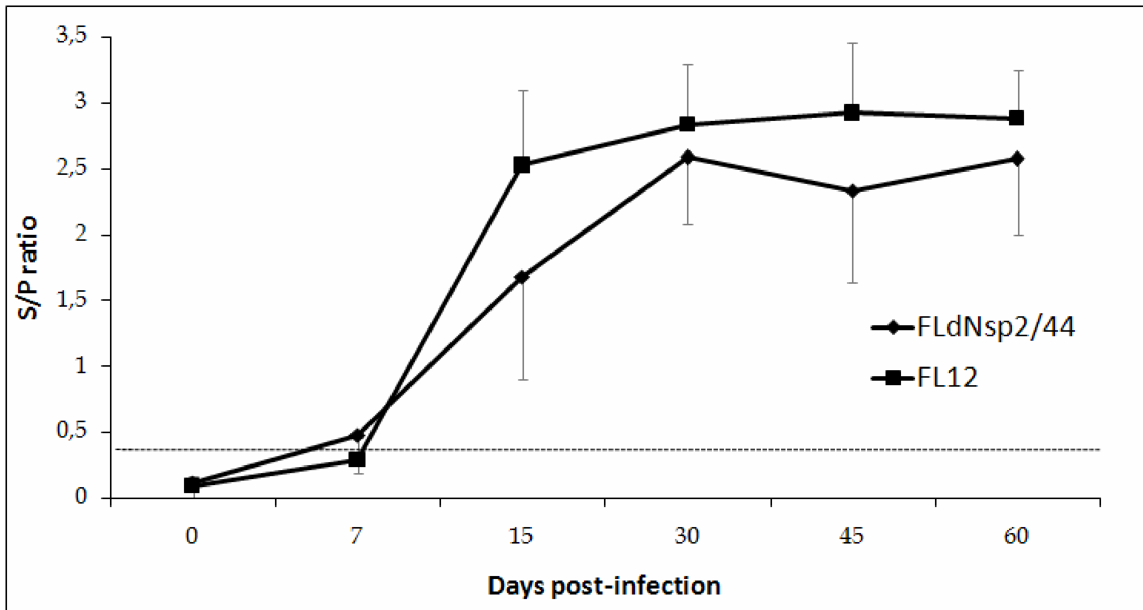

Fig. 2. Serum antibody titers in piglets experimentally inoculated with PRRSV FL12 strain (wild type virus) or FLdNsp2/44 (epitope deletion mutant). S/P ratios were expressed according to a commercial IDEXX ELISA kit and the mean values obtained from the serum samples collected at 0, 7, 15, 30, 45 and 60 days post-infection from the 15 piglets infected in each group are shown. Horizontal bars represent the standard deviation. A dashed line at 0.4 S/P ratio corresponds to the threshold value above which samples are considered positive for PRRSV-specific antibodies.

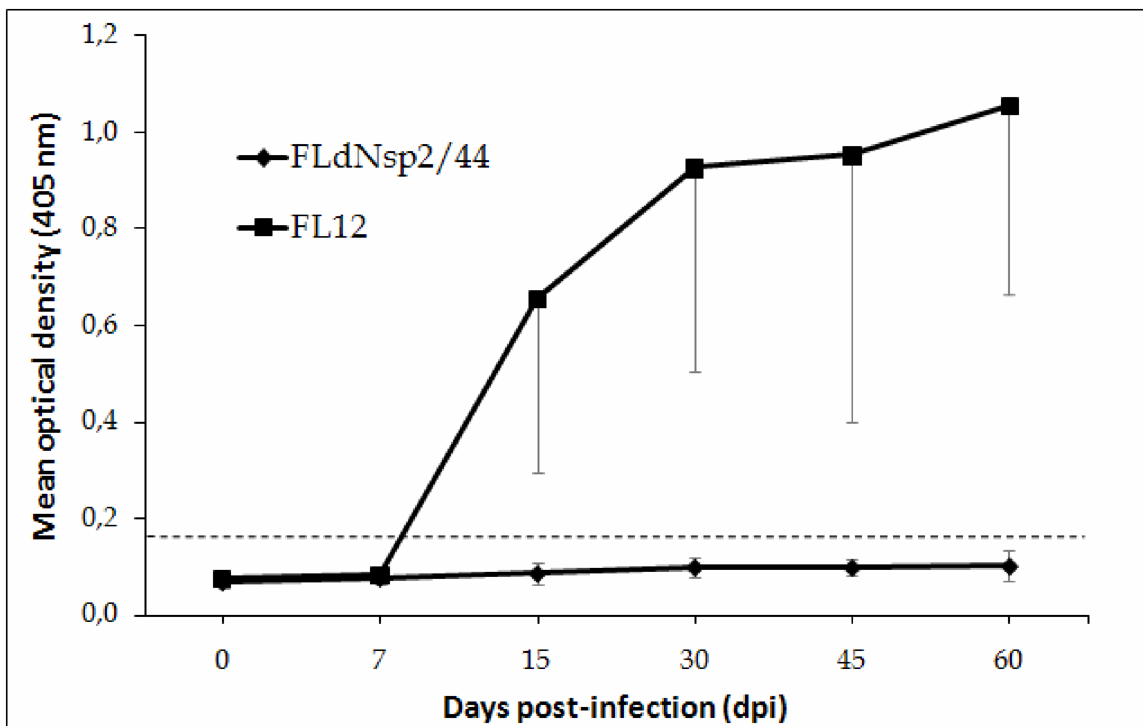

Fig. 3. Serological response of pigs experimentally infected with 10^5TCID$_{50}$/ml of FL12 (wild type) or FLdNsp2/44 (epitope deletion mutant) from day 0 to 60 post-infection. The values represent the mean optical density obtained in a peptide-based ELISA where plates were coated with a 15-mer peptide corresponding to the deleted region in FLdNsp2/44. Horizontal bars indicate the standard deviation and a dashed line corresponds to the cut-off point.

Table 1. Viremia in 15 pigs experimentally infected with 10^5 $TCID_{50}$/ml of FL12 (wild type) or FLdNsp2/44 (epitope deletion mutant). Infectivity is expressed as mean $\log_{10}$ PRRSV titer $TCID_{50}/ml^{-1}$ in the sera of the 15 experimentally infected pigs from days 4 to 30 post-infection ± the standard deviation.

Group	4dpi	7dpi	10dpi	15dpi	30dpi
FLdNsp2/44	2.88 (±0.38)	3.32 (±0.22)	2.55 (±0.46)	2.29 (±0.42)[1]	ND[2]
FL12	3.86 (±0.43)	4.28 (±0.49)	4.25 (±0.38)	3.41 (±0.20)	ND

[1] 13 out of 15 pigs showed detectable levels of virus in sera
[2] ND=Not detectable (< $10^{1.7}$ $TCID_{50}$/ml)

Table 2. *In vivo* phenotype of the wild-type virus (FL12) and epitope deletion mutant (FLdNsp2/44) assessed in a reproductive failure model in pregnant sows (90 days of gestation). The viability scores of offspring at birth and 15 days after farrowing are indicated.

Group	Sow #	Offspring	Viability at birth		Viability at 15 days
			dead	live	live
FLdNsp2/44	1	18	18	0	0
	2	14	11	3	2
FL12[1]	1	16	13	3	0
	2	14	13	1	0

[1] Data obtained from a previous experiment (Kwon et al., 2006)